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Biochimica et Biophysica Acta 1773 (2007) 954–969

www.elsevier.com/locate/bbamcr

Modulation of apolipoprotein D expression and translocation under specific stress conditions

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Received 19 January 2007; received in revised form 5 March 2007; accepted 6 March 2007

Available online 24 March 2007

Abstract

Apolipoprotein D is a lipocalin, primarily associated with high density lipoproteins in human plasma. Its expression is induced in several pathological and stressful conditions including growth arrest suggesting that it could act as a nonspecific stress protein. A survey of cellular stresses shows those causing an extended growth arrest, as hydrogen peroxide and UV light increase apoD expression. Alternatively, lipopolysaccharide (LPS), a pro-inflammatory agonist showed a time- and dose-dependent effect on apoD expression that correlates with an increase in proliferation. At the promoter level, NF- κ B, AP-1 and APRE-3 proved to be the elements implicated in the LPS response. Colocalization of apoDh–GFP fusion constructs with DNA and Golgi markers, immunocytochemistry of the endogenous protein and cell fractionation showed that both serum starvation and LPS treatment caused a displacement of apoD localization. In normal conditions, apoD is mainly perinuclear but it accumulates in cytoplasm and nucleus under these stress conditions. Since nuclear apoD appears derived from the secreted protein, it may act as an extracellular ligand transporter as well as a transcriptional regulator depending on its location. This role of apoD inside the cell is not only dependent of endogenous apoD but may also be provided by exogenous apoD entering the cell.

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Keywords: Apolipoprotein D; Growth arrest; Inflammation; Lipocalin; Nuclear translocation

1. Introduction

Apolipoprotein D (apoD) is a member of the lipocalin superfamily that is found associated with high-density lipopro-

teins in the plasma of humans and other species [1–6]. Unlike most of the other plasma apolipoproteins whose expression is limited to the liver and/or the intestine, apoD, a 29-kDa glycoprotein, is expressed in almost all tissues that have been tested [5,7–13] both during adulthood and during embryonic or postnatal development [14,15]. Although apoD has been shown to bind a number of small hydrophobic molecules, the physiological ligands of apoD have yet to be definitively identified, and it has been proposed that apoD may have multiple tissue-specific, physiological ligands and functions [9,16,17].

In cultured cells, apoD expression is modulated in response to various stimuli. It can be enhanced by growth arrest and senescence [18,19] or by other factors such as steroids [20–22], interleukin-1 α [23], 1,25-dihydroxyvitamin D3 [24], retinoic acid [24,25], or 25-hydroxycholesterol [26]. The promoter region of the human apoD gene contains several regulatory elements including serum response elements (SRE) and an alternating purine–pyrimidine stretch implicated in the response to growth arrest [19]. It also contains acute phase, estrogens,

Abbreviations: apoD, apolipoprotein D; AP-1, activating protein 1; APRE, acute phase responsive element; BFA, Brefeldin A; BrdU, Bromodeoxyuridine; C/EBP, CCAAT/enhancer binding protein; CPT, camptothecin; ERE, estrogen responsive element; FGF, fibroblast growth factor; GRE, glucocorticoid responsive element; HDL, high density lipoprotein; LPS, lipopolysaccharide; MOPS, 3-[N-morpholino] propane-sulphonic acid; MTS/PMS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/phenazine methosulfate; NF- κ B, nuclear factor kappa B; PRE, progesterone responsive element; SDR, sterol-dependent repressor; SRE, serum responsive element; STAT3, signal transducer and activator of transcription 3; TUNEL, Terminal deoxynucleotidyl Transferase-mediated dUTP nick end labeling

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progesterone, glucocorticoids, fat, thyroid-hormone, metal and stress response elements (APRE, ERE, PRE, GRE, FSE, TRE, MRE, STRE), sterol-dependent repressor (SDR), activation proteins 1 and 2 (AP-1, AP-2), and nuclear factor kappa B (NF- κ B) binding sites [19]. There seems to be a significant biological role for a tight physiological regulation of apoD, and an altered apoD expression has been reported in several pathological conditions.

ApoD is expressed at high levels in the normal central and peripheral nervous systems of various species [5,7–10,27]. ApoD levels are further increased in human neuropathologies such as Alzheimer's disease, stroke, meningoencephalitis, motor neuron disease, dementia [28], Niemann–Pick [29], schizophrenia [30,31], Parkinson disease [32] and multiple sclerosis [33]. ApoD is also increased in several animal models of brain injury [34–39]. In non-neurological pathologies, apoD protein accumulates in atherosclerotic plaques [40], in the cyst fluid from women with breast gross cystic disease [41,42], in various cancers and in metabolic diseases such as non-insulin-dependent diabetes mellitus type II, obesity, and syndrome X [for review, see [43]].

Other than the increase of apoD expression, these conditions share the presence of inflammation, oxidative stress and apoptosis. Inflammation is a physiological response to tissue injury, trauma, or infection and consists of a systemic reaction to fight further tissue damage, destroy infective organisms and activate repair processes. Chronic inflammation is associated with certain metabolic diseases and cancers. There is also an inflammatory component in Alzheimer's disease, multiple sclerosis, schizophrenia, traumatic brain injury and other neuropathologies [44]. The long-term administration of non-steroidal anti-inflammatory drugs (NSAIDs) or cyclooxygenase (COX) inhibitors appears to reduce the incidence, or delay the onset, of Alzheimer's disease [45]. Both naproxen and ibuprofen (2 NSAIDs) inhibit the aggregation of β -amyloid peptides to senile plaques *ex vivo* [46]. Also, apoE may play an isoform-specific role in mediating the systemic and brain inflammatory responses. ApoE4 has been associated with the early onset of Alzheimer's disease and poor prognosis in other disorders such as multiple sclerosis [47,48]. ApoD levels in CSF and hippocampus increased as a function of inheritance of the apoE4 allele [28]. Mice expressing the apoE4 allele and injected with LPS have significantly greater systemic and brain elevations of the pro-inflammatory cytokines TNF α and IL-6 as compared with their apoE3 counterparts [49]. Furthermore, oxidative modification of lipids occurs during inflammatory processes and leads to the formation and accumulation of biologically active lipid oxidation products that induce specific cellular reactions such as apoptosis. Moreover, oxidative stress has long been associated with the neuronal cell death in some neurodegenerative conditions. It is still unclear whether oxidative stress is the initiating event or a downstream consequence of the neurodegenerative process in Alzheimer's disease and multiple sclerosis, two degenerative diseases presenting apoD overexpression. The oxidant and antioxidant defense system may also be dysfunctional in schizophrenia and bipolar disorders, which also show apoD induction [30]. Finally, oxidative stress resulting from an

imbalance between pro-oxidants and anti-oxidants seems to play an important role in human breast carcinogenesis [50]. Apoptosis has also been reported in situations showing apoD induction as kainate excitotoxicity, entorhinal cortex lesioning and traumatic brain injury [37–39].

The heterogeneity of situations triggering apoD expression, although informative about the importance of apoD, leaves us in the dark about the primary event responsible for its induction. Thus, any stress situation may initiate apoD expression and it has been suggested that apoD is a nonspecific response to different stimuli and may be part of an antioxidant defense system [51].

The aim of this study is to determine which cellular stresses affect apoD expression and their effect on cell viability, apoptosis and senescence. The incidence of some of these stresses on apoD protein synthesis and localization is further examined. The responsive elements at the promoter level are also explored. Our results show that only those stresses causing an extended growth arrest increase apoD expression. Interestingly, lipopolysaccharide (LPS), a pro-inflammatory agonist in fibroblasts showed a time- and dose-dependent effect on apoD expression that correlates with an increase in proliferation. At the promoter level, NF- κ B, AP-1 and APRE-3 proved to be the responsive elements. LPS exposure and growth arrest also induce a translocation of apoD to the nucleus.

2. Materials and methods

2.1. Cell culture

All cells were obtained from ATCC, Rockville, MD and maintained in DMEM (Invitrogen) supplemented with 10% calf serum (NIH/3T3), in MEM (Invitrogen) supplemented with 10% fetal calf serum (HepG2, HeLa and 293), in RPMI (Invitrogen) supplemented with 10% fetal calf serum (U373MG and U87). All cells were also supplemented with penicillin G (100 units/ml) and streptomycin (100 μ g/ml), maintained at 37 °C in a 5% CO₂ humidified atmosphere and were fed every 2 days with fresh medium. For the analysis of gene expression in sparsely growing cultures (normal condition), cells were maintained in medium supplemented with 10% calf serum and harvested at 50% of confluence. For analysis of growth arrest, cells were incubated in 0.2% serum when they reached 80% of confluence. Medium was changed after 24 h and cells were harvested after 4 days.

2.2. Stress induction

Cells were plated at 50% confluency, allowed to recover 24 h then stressors were applied as follows. Two heat and two cold shocks, one of high and another of low intensity, were used. Cells were incubated at 42 °C for 5 h (low heat) or at 45 °C for 20 min (high heat). Cells were also exposed to 32 °C for 4 h (low cold) or to 4 °C (high cold) for 20 min. Cells were always maintained in a humidified atmosphere containing 5% CO₂ except for the 4 °C condition where culture plates were tightly wrapped with parafilm and refrigerated for 20 min. Cells were allowed to recover for 6 h at 37 °C before analysis. For hypo-osmotic shock, cell medium was reduced to 60% normosmotic strength by addition of sterile deionized water. Cells were exposed to this hypo-osmotic condition during 2 h at 37 °C then washed with PBS and replaced in normosmotic medium for 6 h before harvest. For hyper-osmotic conditions, cells were maintained for 4 days in regular growth medium supplemented with 150 mM NaCl before analysis and the medium was changed every day. Metal stress was caused by addition of metal salts to cells (100 μ M ZnCl₂, 100 μ M CaCl₂, 50 μ M MnCl₂, 100 μ M MgCl₂, 50 μ M Alk (SO₄)₂, 50 μ M LiCl) for 4 h at 37 °C. After this treatment, cells were washed and were allowed to recover in fresh growth medium for 4 h at 37 °C. Oxidative stress was induced by, unless otherwise indicated, addition of 100 μ M or 300 μ M H₂O₂ (Sigma-Aldrich) to the cells for 2 h at 37 °C and cells

were let to recover for 48 h after growth medium was replaced. Rose Bengal stress was produced by irradiating media containing 1 μ M Rose Bengal with a 500 W lamp from a fixed distance of 66 cm for 15 min. To induce apoptosis, cells were incubated for 16 h at 37 °C with 5 or 10 μ M of camptothecin (CPT, Sigma-Aldrich) or for 8 h with 0.1 μ M staurosporine (Sigma-Aldrich) in serum free DMEM. For UV irradiation, medium and plate lids were removed and cells were irradiated with a Phillips® Sterilamp (256 nm, 15 W) with a source-to-target distance of 70 cm. Then, cells were incubated for an additional 16 h at 37 °C. For LPS treatment, cells were exposed to medium containing 2, 5, or 10 μ g/ml of LPS (Sigma-Aldrich) for 4 days or as indicated. Medium was refreshed every day. Alternatively, cells were also exposed to 10 ng/ml or 100 ng/ml of bFGF or a combination of LPS 5 μ g/ml, diclofenac 200 μ M and bFGF (Sigma-Aldrich).

2.3. RNA extraction and northern blot analysis

For each condition, cells were washed in PBS, directly lysed in the culture dish by adding TRIZOL Reagent (Invitrogen) as recommended by the supplier and kept at –80 °C until all samples were collected. For northern blots, 20 μ g of total RNA was denatured in formaldehyde/formamide and migrated in 1.5% agarose gel containing MOPS (20 mM) and formaldehyde (17%). Nucleic acids were transferred to Osmonics Nylon Transfer Membranes (Fisher Scientific) and UV-fixed for 3 min. The membranes were hybridized with [α -32P] dCTP-labeled apoD, hsp70, caveolin-1 or GAPDH cDNAs, exposed to Biorad Imaging screen K and revealed with a PhosphorImager (Biorad Molecular Imager FX) and Quantity One software (Biorad). For each value, the optical density measured for each gene tested was divided by that of the GAPDH mRNA. The ratio obtained from sparse cultures was given an arbitrary value of one.

2.4. TUNEL assay

Apoptosis was monitored by TUNEL reaction using the *In situ* cell detection kit, fluorescein labeled (Roche, Mannheim, Germany) according to the manufacturer's instructions. Cells were plated on 4 well-Sonic Seal Slides (Nunc, VWR) at 50% confluency, allowed to recover 24 h then subjected to stress conditions as described above. Results are expressed as percentage of fluorescent apoptotic cells.

2.5. MTS/PMS viability test

Cell viability was measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Cells were plated as above and the results are expressed as percentage of proliferative cells.

2.6. BrdU incorporation

The proliferation assay was performed using colorimetric BrdU labeling and detection kit I according to the manufacturer's protocol (Roche, Mannheim, Germany). Results are expressed as BrdU incorporating cells relative to that of growing unstressed cells.

2.7. Beta-galactosidase staining of senescent cells

The β -galactosidase activity at pH 6.0, a known characteristic of senescent cells, was used to measure senescence. Cells were plated as above and the results are expressed as percentage of β -galactosidase expressing cells.

2.8. Construction and transfection of apoDh–GFP fusion protein expression vectors

The human apoD coding sequence, including the signal peptide, was cloned in frame in pEGFP-N1 and pEGFP-C1 vectors (BD Biosciences Clontech). The construct was transfected in NIH/3T3 cells with Qiagen's Polyfect transfection reagent as recommended by the supplier. Twenty-four hours later, cells were rinsed twice with phosphate-buffered saline and culture medium was changed for medium containing 10% serum supplemented with 5 μ g/ml of LPS or low-serum medium (containing 0.2% serum) or normal medium containing 10% serum.

2.9. Immunocytochemistry and cytochemistry

Cells were fixed with 4% paraformaldehyde 0, 3 or 6 days after stress induction and stained with propidium iodide (Sigma-Aldrich) or bodipy ceramide TR (Invitrogen). All fluorescent imaging was done at 40 \times magnification using confocal microscopy. For control, cells were transfected with pEGFP-N1 vector alone. For immunofluorescence staining, NIH/3T3 or U373MG cells were grown on Labtek chamber slides (Fisher Scientific), subjected to stress conditions as above and fixed with 4% paraformaldehyde for 10 min at room temperature. After five washes with PBS, fixed cells were incubated in blocking and permeabilization solution (3% serum from the host animal of the secondary antibody and 0.2% Triton X-100 in PBS) for 1 h. Cells were sequentially incubated with primary antibody (polyclonal anti-mouse apoD raised against the bacterially expressed GST-fused mature mouse apoD, 1:50, Car2 polyclonal anti-human apoD raised against human apoD purified from mammary cyst fluid, 1:50, or 2B9 monoclonal anti-human apoD, 1:200 [28]) and then corresponding fluorophore-labeled secondary antibodies (Cy3- and FITC-labeled anti-rabbit IgG (Cedarlane laboratories limited), 1:100, for anti-human apoD and anti-mouse apoD polyclonal antibodies respectively and FITC-labeled anti-mouse IgG (Cedarlane laboratories limited), 1:200, for anti-human apoD monoclonal antibody) for 1 h, respectively. Cells were washed five times with PBS and then mounted with Slowfade Gold Antifade (Molecular Probes) before imaging. For inhibition of protein secretion by brefeldin A treatment, apoDh–GFP transfected cells were grown on chamber slides and subjected to stress conditions during 4 days. Cells were then treated with or without brefeldin A (5 μ g/ml) (Sigma-Aldrich) for 1 h and fixed 24 h later for confocal microscopy.

2.10. Human apoD promoter-luciferase constructs and mutagenesis

Heterologous luciferase (LUC) reporter gene constructs containing different portions of the human apoD gene promoter were made by classic methods as previously described [19]. The APRE-3, the two NF-kB and the AP-1 elements present in region –816 to –471 were mutated by oligonucleotide-directed PCR mutagenesis (Table 1). For that purpose, PCR-amplified –816/–471 portion of

Table 1
Sites implicated in apoD transcription induction following LPS exposure

Element	Sequence	Position	Orientation	Primers used for mutagenesis
APRE-3	AACTTTTAGCCCCAGTT	–681 to –665	(+)	Forward: 5'-CTCAACTTTcgGCCaaAGTTGTTAGA-3' Reverse: 5'-TCTAACAACttGGCcgAAAGTTGAGA-3'
NF-kB1	ACTGGTATCTCCCT	–656 to –642	(–)	Forward: 5'-ACTGGTATCTaaCTACACGTCTGGC-3' Reverse: 5'-GCCAGACGTGTAGttAGATACCAGT-3'
NF-kB2	GGGGGCTGCCC	–568 to –558	(+)	Forward: 5'-GAGAAGTTAGtttGCTGCCcAGG-3' Reverse: 5'-CTGGGcAGCaaCTAACTTCTC-3'
AP-1	TTGACTCAT	–535 to –527	(+)	Forward: 5'-CAGCTTTGACTtgTAACCCTCGC-3' Reverse: 5'-GCGAGGGTTAcaAGTCAAAGCTG-3'

The primers used for mutagenesis of the region –816/–417 cloned upstream of the minimal promoter –179/–4 are indicated. The mutated nucleotides are indicated in lower case.

the apoD promoter was cloned in the correct orientation upstream of position –179 upstream of the luciferase reporter gene in the pXP2 expression vector. Eight complementary mutated primers and two primers from the vector were used in two-step PCR (Table 1). Multiple mutants were constructed by the same technique using sequential mutagenesis. All of the luciferase reporter constructs were sequenced to confirm their integrity. Each construct was co-transfected in NIH/3T3 cells with a β -galactosidase expression vector with Polyfect reagent. After transfection, the cells were maintained in either LPS containing (5 μ g/ml) or LPS-free medium. The luciferase activity was analysed after 4 days, and the results were normalized for the β -galactosidase activity.

2.11. Luciferase and β -Galactosidase assays

Cells were washed with PBS before adding 100 μ l of Tris–HCl, 0.25 M, pH 7.8. Cells were then scraped with a cell lifter, transferred into microcentrifuge tubes, lysed by three cycles of freeze–thawing and the lysates were stored at -20°C as described previously [19]. Luciferase assays were performed with a Wallac 1404 luminometer using the conditions and buffers recommended by the Luciferase assay system (Promega). β -Galactosidase assays were done as follows: each sample (30 μ l) was adjusted to a final concentration of 1 mM MgCl_2 , 45 mM β -mercaptoethanol, 0.88 mg/ml *o*-phenyl- β -D-galactopyranoside, and 0.1 M sodium phosphate (pH 7.5) and incubated at 37°C for 30 min. Reactions were stopped by the addition of Na_2CO_3 to a final concentration of 625 mM, and the optical density was read at 420 nm. The results were standardized by calculating promoter activity relative to that of the co-transfected internal control plasmid pRSV β GAL. Each value is the average of at least three independent experiments performed in triplicate.

2.12. Extraction of total and nuclear proteins

For total protein extraction, 2.5×10^5 cells were lysed in 10 μ l lysis buffer (50 mM Tris–HCl, pH 7.3, 150 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, and 10% CompleteTM protease inhibitors (Roche, Indianapolis, IN)). After 30-min incubation at 4°C , lysates were sonicated and cleared by 10 min of centrifugation at $20,000 \times g$ at 4°C . For nuclear protein extraction, cells were washed twice with PBS and lysed in a solution containing 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl_2 , 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40. Nuclei were pelleted by centrifugation at $2000 \times g$ for 2 min and washed once in the same buffer to remove residual cytoplasmic proteins. After removal of cytoplasmic proteins, cell nuclei were lysed at 37°C for 3 to 5 min in a hyperosmolar neutral-pH solution with 0.5 M NaCl, 50 mM MgCl_2 , 2 mM CaCl_2 , 10 mM Tris buffer, 100 U of DNase I/ml, and 10% CompleteTM protease inhibitors. The protein concentration was determined spectrophotometrically at 590 nm using the Bio-Rad protein assay reagents (Bio-Rad Laboratories). All extracts were stored at -70°C .

2.13. Immunoblotting

Protein extracts (10 μ g) were heated for 10 min at 70°C before being loaded onto a 12% SDS-polyacrylamide gel. A prestained size marker (Biorad prestained SDS-PAGE standard, low range) was included in each run. The gel was subjected to electrophoresis for 1 h at 40 mA and then transferred by electroblotting to a polyvinylidene difluoride (PVDF, Millipore) membrane for 45 min at 300 mA. Membranes were blocked for 1 h at room temperature with blocking buffer (PBS containing 0.2% Tween 20 and 5% skim milk powder) and incubated for 1 h at room temperature with 1:1000 anti-human apoD (Caro2 polyclonal antibody), 1:1000 anti-GFP (Chemicon) or 1:4000 anti-GAPDH (Calbiochem) or overnight at 4°C with 1:1000 anti-mouse apoD polyclonal antibody in blocking buffer. Subsequently, the blots were incubated under gentle agitation at room temperature with a secondary horseradish peroxidase conjugated anti-rabbit antibody (GE Healthcare) diluted 1:5000 in blocking buffer. The blots were developed using the enhanced chemiluminescence method (Amersham-Pharmacia) with X-ray film.

2.14. Statistical analysis

Statistical significance of the apoD mRNA expression, apoptosis, cell viability and senescence levels in cells subjected to different stress in

comparison with normal conditions was evaluated using an unpaired Student's *t*-test or a one-way ANOVA. Statistical differences between the different constructs used for the identification of LPS responsive elements in the apoD promoter were assessed by ANOVA. Differences identified by ANOVA were pinpointed by the Turkey–Kramer multiple comparison test.

3. Results

3.1. ApoD expression in response to cellular stress

Previous studies have shown that apoD mRNA is induced in cell cultures under specific conditions [for review, see 6, 43] and that its expression could be part of an antioxidant defense system [51]. Tatil, the plant apoD homolog is upregulated in response to temperature and water stress [52]. However, in mammalian cells, no data are available regarding temperature

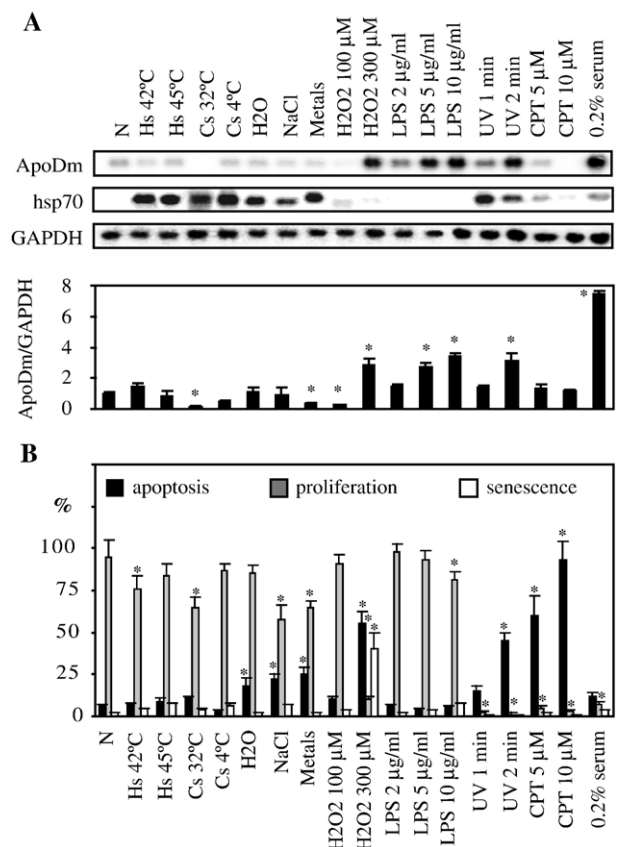


Fig. 1. ApoD mRNA modulation under stress conditions is associated with variations in proliferation, senescence and apoptosis. (A) ApoD mRNA expression in NIH/3T3 cells after exposure to various stresses. Untreated growing cultures (N) and serum-starved cultures (0.2%) are included as controls. The following lanes are, respectively, RNA from cultures exposed to high temperatures (Hs 42°C and Hs 45°C), low temperatures (Cs 32°C and Cs 4°C), hypo- and hyper-osmotic conditions (H_2O and NaCl), metal salts, hydrogen peroxide (H_2O_2 100 μM and 300 μM), lipopolysaccharide (LPS 2, 5 and 10 $\mu\text{g/ml}$), UV light (1 and 2 min) or camptothecin (CPT 5 and 10 μM). Blots were hybridized with mouse apoD, hsp70 and GAPDH cDNAs. (B) Apoptosis, viability and senescence were measured by the TUNEL assay, by the MTS/PMS mitochondrial respiration assay and by the β -galactosidase activity, respectively. Data are represented as mean \pm S.D. of at least three experiments. Asterisk indicates statistical difference compared to normal conditions (unpaired Student's *t*-test, $*p < 0.001$).

or osmolarity stress. Also, no direct evidence of a relation between apoD and apoptosis, oxidative stress or inflammation has been reported. To analyze this aspect, we measured apoD mRNA expression in murine NIH/3T3 fibroblasts exposed to various stresses (Fig. 1A). Our team previously used these cells since they express apoD in a fashion similar to glial, epithelial and primary fibroblasts [19]. Endogenous apoD mRNA expression was very low in growing cells and in cells exposed to heat and cold shocks of high (Hs 45 °C, Cs 4 °C) or low (Hs 42 °C, Cs 32 °C) intensity. ApoD mRNA expression was also very low in cells grown in hypo- or hyper-osmotic conditions. Since the apoD promoter contains one putative metal responsive element [19], a combination of metals was tested. However, no change in apoD mRNA levels was detected. For these conditions, longer exposures and recovery times were also tested and no further increase of apoD expression was observed. In contrast, H₂O₂ and UV light induced apoD transcripts accumulation in a dose-dependent manner (Fig. 1A). This was also observed with Rose Bengal another inducer of oxidative stress (data not shown). Lipopolysaccharide (LPS) also induces a dose-dependent response. Finally, camptothecin, an apoptosis inducer, had no effect on apoD mRNA expression.

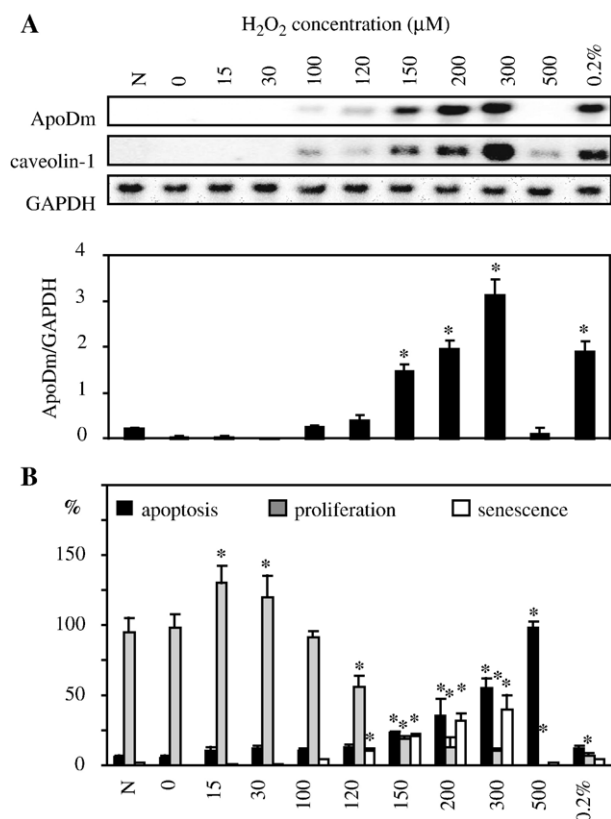


Fig. 2. ApoD mRNA expression after H₂O₂ treatment is concentration-dependent. (A) Total RNAs were isolated from NIH/3T3 untreated (N) or treated with increasing concentrations of H₂O₂. Serum starved cells are included as control. Hybridization was performed with mouse apoD, caveolin-1 and GAPDH cDNAs. (B) Quantification of apoptosis, viability and senescence after H₂O₂ exposure was performed as in Fig. 1B. Data are represented as mean \pm S.D. of at least three experiments. Asterisk indicates statistical difference compared to normal conditions (unpaired Student's *t*-test, **p* < 0.001).

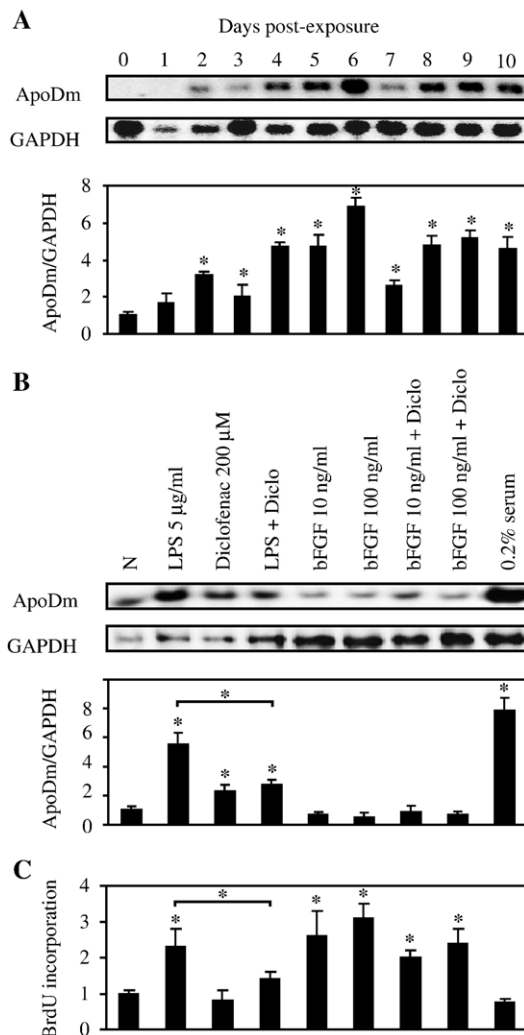


Fig. 3. ApoD mRNA expression after LPS treatment and relation with inflammation and proliferation. (A) NIH/3T3 cells were exposed to LPS (5 μg/ml) during the indicated number of days. Northern analysis was performed with total RNAs probed with mouse apoD and GAPDH cDNAs. (B) ApoD expression in cells treated with LPS diclofenac or basic fibroblast growth factor (bFGF) for 4 days. Untreated growing cells (N) and serum-starved cells (0.2%) were included as controls. Northern analysis was performed with total RNAs probed with mouse apoD and GAPDH cDNAs. (C) Analysis of proliferation by BrdU incorporation in cells treated as in B. Data are represented as mean \pm S.D. of at least three experiments. Asterisk indicates statistical difference compared to normal conditions or as indicated (one-way ANOVA, **p* < 0.001).

As expected, hsp70 is increased in the temperature, osmotic, metal, UV and H₂O₂ treatments revealing that the stress applied were effective. However, hsp70 is not activated by LPS. For comparison, serum starved cells were included and they show a substantial apoD mRNA increase as previously reported [19] (Fig. 1A).

It is interesting to note that, with the exception of LPS, those conditions that cause an apoD transcript accumulation also decrease cell proliferation (Fig. 1B). In fact, growing cells and cells exposed to temperature or osmotic shocks, metals or low concentrations of H₂O₂ (100 μM) maintain a proliferative rate above 58% and a low level of apoD mRNA expression. At higher H₂O₂ concentration or long UV exposure, the results may

suggest a relation between apoD transcript accumulation and apoptosis. However, no apoD mRNA induction was observed under treatments with various concentrations and time exposures of camptothecin, (Fig. 1A). Moreover, senescence, which is observed at high H_2O_2 concentration (300 μM), has already

been reported for its ability to induce apoD expression [18]. Finally, the conditions presenting the best induction, LPS exposure and serum starvation, both have apoptosis levels comparable (4% to 6% in LPS) or only slightly superior (12% in serum starvation) to those seen in growing cells (6%). Thus, our

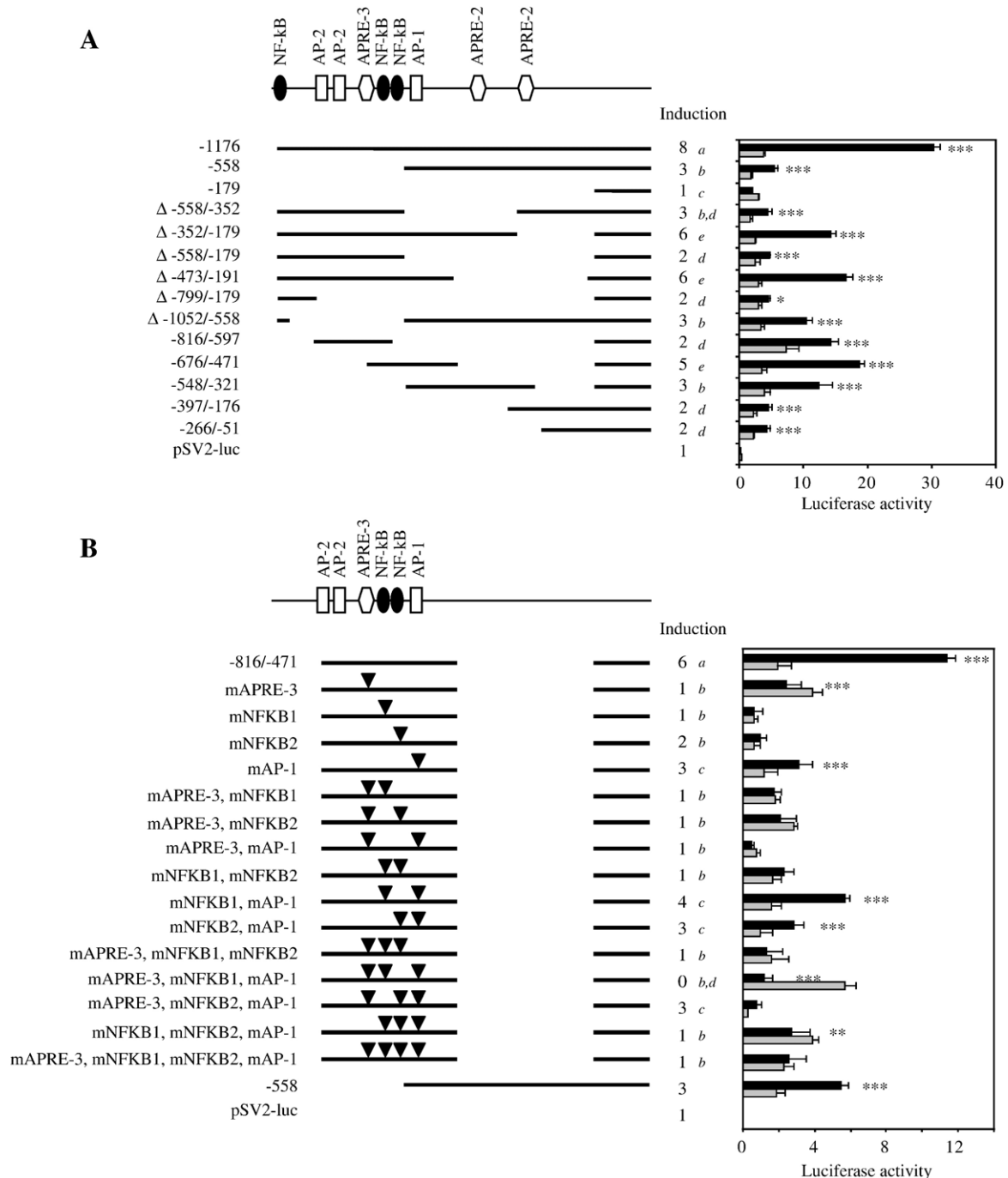


Fig. 4. Identification of LPS responsive elements in the apoD promoter. (A) A series of luciferase reporter constructs was made that contained progressive deletions of the 5'-flanking regions of the apoD gene promoter. (B) Site-directed mutagenesis of APRE-3, NF-kB1, NF-kB2 and AP-1 responsive elements. Each element was mutated alone or in combination with the others in a construct containing the -816/-471 region of the promoter cloned upstream of the minimal promoter -179/-4. The mutated sequences are summarized in Table 1. All constructs were transfected into NIH/3T3 cells, which were maintained in media with (black bars) or without (gray bars) 5 $\mu g/ml$ LPS. Luciferase activities were assayed 4 days after transfection. The induction represents the ratio of luciferase activity in cells treated with LPS to that of cells without LPS. Each value represents the mean \pm S.D. of at least three experiments performed in triplicate (One-way ANOVA, * p < 0.05, ** p < 0.01, *** p < 0.001). Letters at the right of the induction numbers represent statistical significance; different letters indicate statistically different-fold induction.

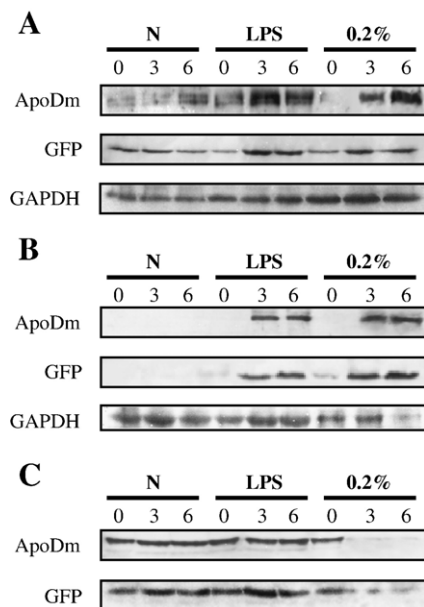


Fig. 5. Effects of LPS exposure and serum starvation on the intracellular localization of endogenous apoD and apoDh–GFP. NIH/3T3 cells were kept in normal growing conditions (N), treated with 5 µg/ml LPS or grown in low-serum medium (0.2%) for 0, 3, or 6 days and then subjected to Western blot analyses. Total (A), nuclear (B), and secreted (C) fractions of endogenous apoD and apoDh–GFP were detected with an anti-mouse apoD (apoDm) and an anti-GFP antibody, respectively. An anti-GAPDH antibody was included as a loading control. Each experiment was repeated at least 3 times.

results clearly indicate that the important inducer of apoD mRNA accumulation is cellular growth arrest rather than apoptosis (Fig. 1B).

3.2. ApoD expression under oxidative stress correlates with decreased cell proliferation

To further assess the contribution of oxidative stress in the apoD mRNA expression observed in Fig. 1A, NIH/3T3 cell cultures were exposed to increasing concentrations of H₂O₂. ApoD transcripts accumulate from 100 µM to 300 µM in a dose-dependent manner (Fig. 2A). This apoD increase correlates well with a decrease in cell viability rather than an increased apoptotic rate (Fig. 2B). Indeed, no apoD accumulation is detected in low H₂O₂ concentrations already documented as being mitogenic (5 µM to 30 µM) [53] nor in the highly apoptotic 500 µM concentration. ApoD induction could also be caused by an increased senescence, which reach 40% in the 300 µM dose, although this situation is closely linked to a growth arrested state. Caveolin-1 is included as a control since its expression has been

demonstrated to be upregulated by H₂O₂ treatment [54]. This inductive effect of H₂O₂ on apoD expression was also observed at lower doses, in a dose-dependent manner with HepG2, HeLa, 293, U373MG and U87 cultures (data not shown) indicating that the human apoD is subjected to the same regulation.

3.3. ApoD expression is associated with pro-inflammatory stimuli

Since the inductive effect of LPS on apoD transcription is dose-dependent (Fig. 1A), we decided to determine whether this effect was time-dependent. LPS induces a time-dependent transcript accumulation during 6 days and reaches a plateau (Fig. 3A). The decrease at day 7 coincided with a partial detachment of the cell monolayer. This inductive effect of LPS on apoD expression was also observed with HepG2, HeLa, 293, U373MG and U87 cultures although the LPS concentrations required to induce apoD expression were lower (data not shown).

Besides its pro-inflammatory properties, LPS has been extensively used as a potent inducer of proliferation and cytokine production in human T-lymphocyte, monocytes/macrophages, vascular cells, polymorphonuclear cells, and even B lymphocytes [55]. To determine which of the two LPS properties is responsible of apoD induction, we analyzed another proliferative molecule, basic fibroblast growth factor (bFGF) and an anti-inflammatory drug, diclofenac. Diclofenac has been previously used in fibroblast cultures to oppose inflammatory effects of LPS and it has an anti-proliferative effect [56]. The bFGF failed to induce apoD (Fig. 3B) whereas LPS and serum starvation showed a good increase in apoD mRNA expression, as expected (Figs. 1A and 3A). Furthermore, the proliferative effect of LPS was similar to that of bFGF with a 2 to 3 fold increase as documented by BrdU incorporation (Fig. 3C). The addition of diclofenac to the culture media slightly increases apoD transcript accumulation. This is most likely due to its anti-proliferative properties. Moreover, diclofenac decreases the proliferative action of bFGF but has no further influence on apoD transcription (Fig. 3B and C). More importantly, diclofenac decreases the LPS-induced apoD expression indicating that the pro-inflammatory rather than the mitogenic properties of LPS are responsible for activating apoD expression (Fig. 3B and C).

3.4. ApoD expression is associated with specific elements in the promoter

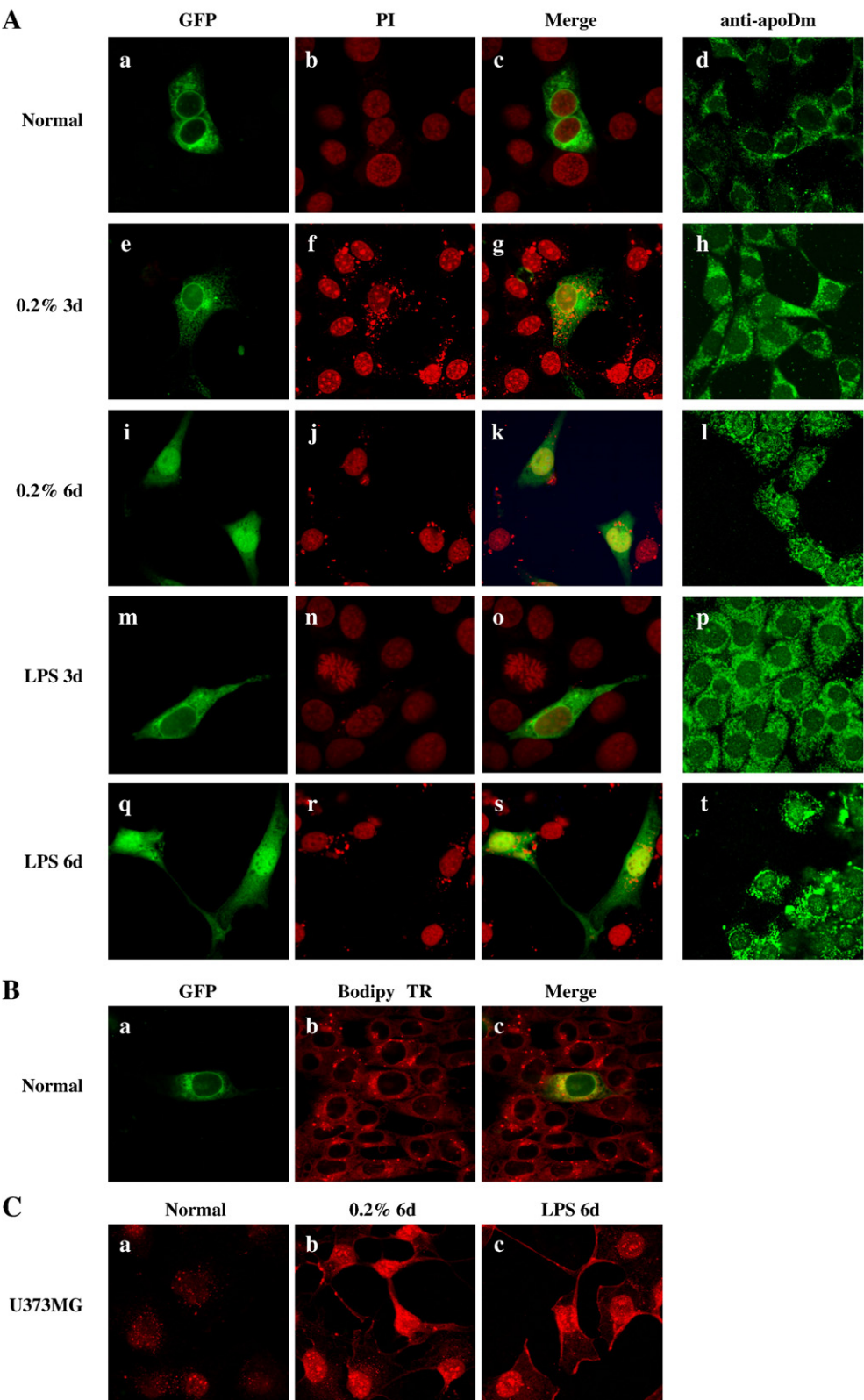
The apoD promoter contains an abundance of potential regulatory elements. It has already been established that a pair

Fig. 6. Intracellular localization of apoD after exposure to LPS or serum starvation. (A) Confocal microscopy analysis of NIH/3T3 cells transfected with the human apoD-coding region fused to GFP (apoDh–GFP) and treated with 5 µg/ml LPS (m–o; q–s) or 0.2% serum medium (e–g; i–k). All fluorescent imaging was done 3 (a–c; e–g; m–o) or 6 (i–k; q–s) days after normal or stress conditions at 40× magnification and each experiment was done at least three times. Green fluorescence (GFP) indicates the position of apoDh–GFP, whereas the red fluorescence (propidium iodide; PI) indicates the position of the nucleus. The extent of nuclear localization of apoDh–GFP is indicated by yellow in the merged images. Results were confirmed by immunofluorescence staining of untransfected cells using a polyclonal mouse apoD antibody in the same stress conditions (d, h, l, p, t). (B) GFP (a), Bodipy TR ceramide (b) and overlaid fluorescence of apoDh–GFP and Bodipy ceramide (c) in NIH/3T3 cells transfected with apoDh–GFP and kept 3 days in normal conditions before being treated with Bodipy TR ceramide. (C) Immunofluorescence staining of human glioma cell line U373MG using a Caro2 polyclonal human apoD antibody. Normal cells (a) were exposed during 6 days to serum starvation (b) or LPS (5 µg/ml; c).

of serum-responsive elements and an alternating purine–pyrimidine stretch are the major determinants of growth arrest-induced apoD gene expression [19]. To identify those elements that regulate apoD expression in fibroblast cultures exposed to LPS, we used deletion mutants containing different

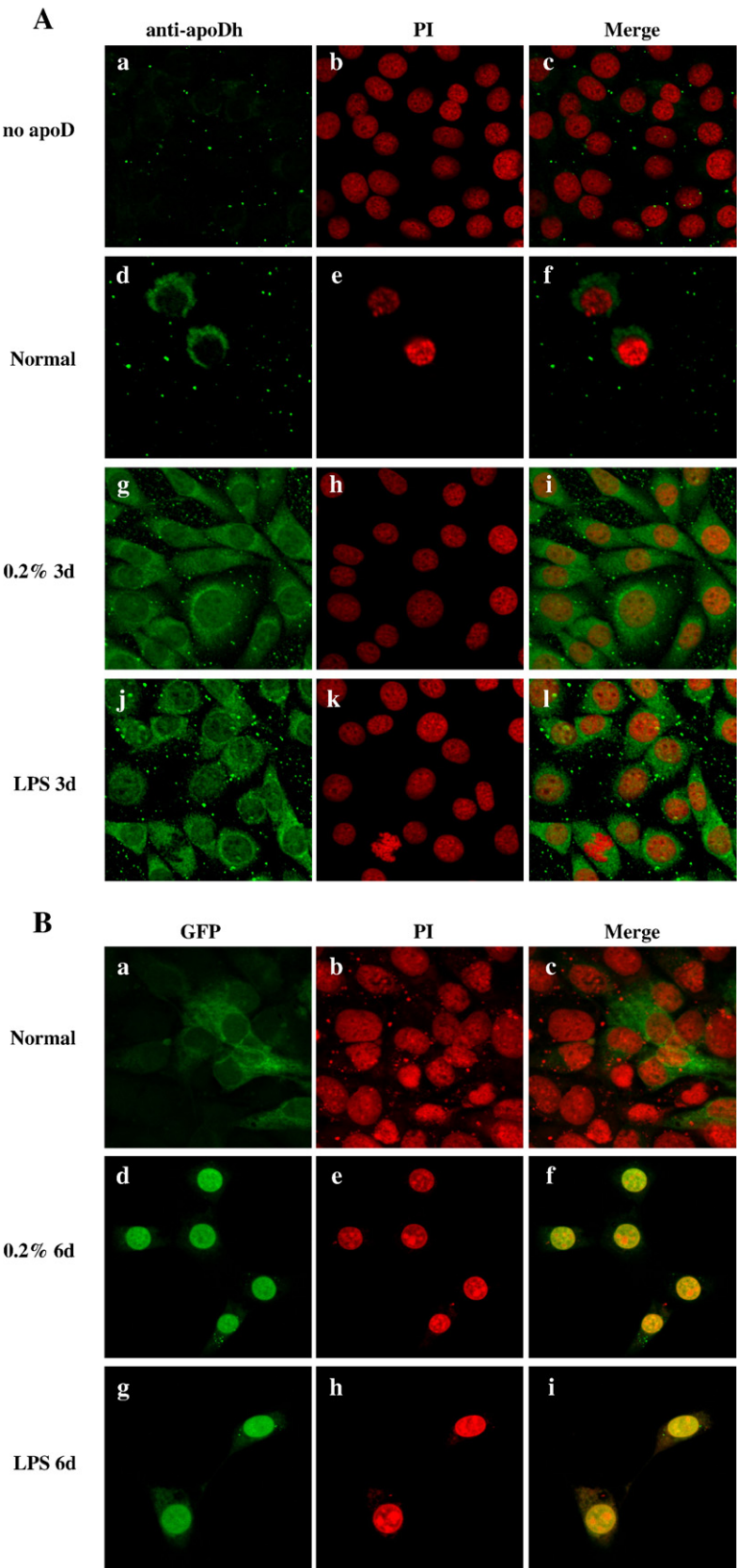
portions of the apoD promoter (–1176 to –4) upstream of the luciferase reporter gene.

With the construct containing the entire promoter region (–1176 to –4), the LPS treatment resulted in an 8-fold induction of promoter activity when compared to normal conditions (Fig.



4A). This induction is significantly reduced to 3-fold when region –1176 to –558 is absent and totally abolished when only the minimal promoter is present (–179). The deletion of either

region –352/–179 or –473/–191 resulted in a small decrease in induction suggesting a negligible contribution of APRE2 elements in response to LPS. This is further confirmed when



comparing constructs –397/–176 and –266/–51 which differ only by one APRE2 element. Furthermore, the deletion of portions –558/–352 and –558/–179 resulted in a drastic reduction of induction (3- and 2-fold respectively) that underlines the importance of AP1 in the LPS response. The induction level can be greatly enhanced by the addition of the AP-1 element (compare deletions –558/–179 and –473/–191). Although important, the AP-1 element alone is insufficient to restore the apoD induction by LPS. Thus, constructs –558 and –548/–321 still display only a 3-fold induction compared to –1176 suggesting that region –1176/–548 is at least as important as the AP-1 element. This region contains two AP2, an APRE3 and two NF- κ B responsive elements. Again, this region alone is unable to restore completely the LPS induction as shown by deletion –558/–179 and construct –816/–597. Thus, it appears that AP-1 cooperates with NF- κ B and possibly with APRE3, as suggested by construct –676/–471 and deletion –473/–191, to induce apoD expression under inflammatory conditions. Finally, comparison of deletion –1052/–558 with fragment –558 reveals the weak importance of the third NF- κ B responsive element at position –1104.

Another construct was thus created that includes all the elements important to LPS response. It contains the region –816/–471 placed upstream of the minimal promoter –179 and presented an activity comparable to that of the –1176/–473 region of the promoter (Fig. 4A). Mutational analysis of the fragment –816/–471 (Fig. 4B) reveals that each of the APRE3, NF- κ B and AP1 elements is important to LPS response and mutagenesis of APRE-3 or NF- κ B1 or NF- κ B2 is sufficient to abolish apoD response to LPS while inactivation of AP-1 only reduces the induction by half. Inactivation of NF- κ B1 or NF- κ B2 reduces apoD expression both in normal conditions and in presence of LPS. This phenomenon was also observed with the NF- κ B element located at position –1104 (compare deletion –1052/–558 with –558 construct in Fig. 4A). Mutation of the APRE-3 increases significantly basal transcription and prevents LPS-induced transcriptional induction. When looking at double and triple mutants (Fig. 4B), the AP-1 mutation counteracts the effect of either NF- κ B mutations but not both suggesting that the interaction between one NF- κ B and the AP-1 element relies on the presence of the other NF- κ B. This is clearly illustrated with the double mutants mNF- κ B1, mAP-1 and mNF- κ B2, mAP-1. However, AP-1 mutation cannot disable the APRE-3 mutation effect although the promoter activity was reduced in both conditions. AP-1 seems to have a different effect on APRE-3 depending on the NF- κ B mutations. When mutated AP-1 and APRE-3 sites are combined with a mutated NF- κ B1 element, the basal promoter activity was increased whereas LPS-stimulated activity was not. This was not the case when the AP-1 and APRE-3 mutations were combined with a mutated NF- κ B2 element.

3.5. ApoD protein localization is altered by serum starvation and LPS treatment

It is now clear that apoD transcription is modulated by specific stress signaling pathways. We then verified the protein induction and localization after LPS exposure and serum starvation-induced growth arrest. For this purpose, an expression vector containing the entire human apoD coding sequence, including the signal peptide, fused to the N-terminal end of the fluorescent GFP protein (apoDh–GFP), was transfected and the fusion protein was followed by western blot analysis and confocal imaging in stressed and unstressed cells (Figs. 5 and 6). As already observed with the transcripts (Figs. 1A and 3A), in total cell extracts, the endogenous mouse apoD increases in a time-dependent manner after exposure to those stresses as detected with a polyclonal antibody against mouse apoD (Fig. 5A; apoDm). However, the protein increase was not as high as for the mRNA and after 6 days of serum starvation, the protein accumulation was 3.5 fold compared to 10 fold for the mRNA [19]. Similarly, after 6 days of LPS exposure, apoD transcript and protein levels are increased 7 fold and 1.5 fold, respectively. It is noteworthy that in addition to its accumulation in the cell, apoD is also found in the nucleus after stress exposure (Figs. 5B and 6). Interestingly, the nuclear apoD has a molecular mass similar to that of the secreted protein (\approx 55 kDa for apoDh–GFP and 30 kDa for endogenous mouse apoD). Finally, it is also clear that apoD is secreted both in normal and LPS conditions. However, endogenous apoD is no longer secreted after 3 and 6 days of serum starvation (Fig. 5C). When growth arrested cells are reallocated to proliferate by the addition of 10% serum to the culture medium, the endogenous mouse apoD returns to basal levels, is no longer found in the nucleus and is secreted again in the extracellular space (data not shown). In the same way, apoD was no longer found in the nucleus when the LPS treatment was interrupted. These changes in localization are also obvious in immunoblots with anti-GFP antibodies (Fig. 5). In total cell extracts (Fig. 5A), apoDh–GFP is detected in all conditions as expected since it is transcribed from the CMV promoter. In nuclear extracts, however, apoDh–GFP is absent in normal conditions, most likely because it is secreted but it is detected after 3 and 6 days after LPS exposure and serum deprivation (Fig. 5B). This localization is directly correlated with that of the endogenous apoD.

The subcellular localization of apoDh–GFP fusion protein was further examined using confocal fluorescence microscopy. In unstressed normal cells, (Fig. 6A, a–c), apoD is found in the perinuclear area. This localization is consistent with the Golgi apparatus as demonstrated by overlaid fluorescence of GFP and Bodipy TR ceramide (Fig. 6B, a–c). Similar to immunoblotting results, no apoDh–GFP is present in the nucleus as established by the lack of colocalization with the

Fig. 7. Internalization of exogenous human apoD or apoDh–GFP. (A) Immunofluorescence staining of cells exposed to purified human apoD using a monoclonal human apoD antibody. Staining was performed 3 days after the addition of human apoD (200 ng/ml) to the culture media of cells in normal (d–f), in low serum (0.2%; g–i) or in LPS (5 μ g/ml; j–l) conditions. As a control, staining was conducted on cells without the addition of human apoD (a–c). Green fluorescence (anti-apoDh) indicates the position of human apoD, whereas the red fluorescence (propidium iodide; PI) indicates the position of the nucleus. The extent of nuclear localization of human apoD is indicated by yellow in the merged images. (B) Confocal imaging of extracellular apoDh–GFP. Conditioned media from cells transfected with apoDh–GFP was added to cells in normal (a–c) or stress conditions (d–i) during 6 days.

nuclear marker propidium iodide (PI) (Fig. 6A, a–c). After 3 days of serum starvation or LPS treatment, this localization is altered. ApoDh–GFP distribution is extended in the cytoplasm and becomes visible in the nucleus (Fig. 6A, e–g and m–o). Interestingly, during serum starvation, apoDh–GFP appears as a dotted localization in the cytoplasm (Fig. 6A, e). The nuclear localization of apoDh–GFP intensifies after 6 days of stress exposure (Fig. 6A, i–k and q–s) as showed by merged images with PI staining of the nucleus. We also produced the reverse fusion protein containing the human apoD coding sequence linked to the C-terminus of GFP (GFP–apoDh). Since the apoD peptide signal is now in the middle of the fusion protein, the fluorescence patterns indicated a subcellular distribution identical to the GFP alone (results not shown). The subcellular distribution of endogenous apoD was also probed with a polyclonal mouse apoD antibody. Confocal microscopy confirmed that apoD is perinuclear in normal conditions (Fig. 6A, d) and translocates to the nucleus as stress is prolonged (Fig. 6A, h, l, p, t). Together, these results indicate that apoD drives GFP into the nucleus. Finally, immunofluorescence staining of human glioma cell line U373MG using a polyclonal human apoD antibody shows that the nuclear localization pattern is not restricted to NIH/3T3 cell line. As a matter of fact, fluorescence is clearly concentrated in the nucleus of cells exposed to serum starvation or LPS conditions during 6 days but appears diffuse in normal conditions.

3.6. Exogenous apoD enters cells and affects cellular status

ApoD is a glycosylated and secreted protein. Its presence in the nucleus might be explained by its re-entry in the cell after secretion and its transport to the nucleus. It has already been shown that human apoD, purified from breast cyst fluid, enters ovine vascular smooth muscle cells when added to the culture medium [40]. Moreover, it interacts specifically with the cytoplasmic portion of the leptin long form receptor Ob-Rb [57]. In the same way, human apoD, purified from cyst fluid, also enters NIH/3T3 cells when added to the culture medium, as detected with human apoD specific antibody (Figs. 7A and 8B). Moreover, the human apoD localization is dependent on cellular status. In normal growing cells, human apoD is detected in the cytoplasm but not in the nucleus (Fig. 7A, d–f). In comparison, in serum starved and LPS exposed cells, human apoD accumulates in the cytoplasm but also in the nucleus as demonstrated by merged images of the fluorescence of the anti-human apoD antibody and the nuclear marker PI (Fig. 7A, g–l). However, in absence of human apoD, no signal could be detected demonstrating the high specificity of the antibody towards human apoD (Fig. 7A, a–c). Similarly, when conditioned media from apoDh–GFP transfected cells was added to cells that were further exposed to normal or stress conditions during 5 days, fluorescence could be detected in the cytoplasm of normal growing cells (Fig. 7B, a–c). In serum starved and LPS exposed cells, the fluorescence accumulated inside the nucleus as demonstrated by a strong colocalization with nucleus staining (Fig. 7B, d–i). The weaker nuclear localization obtained with the addition of human cyst fluid

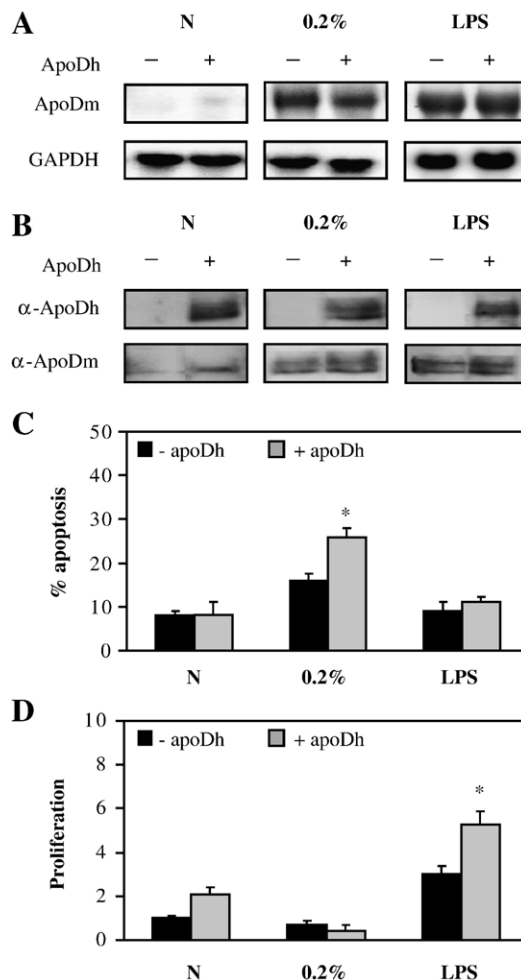


Fig. 8. Effect of exogenous human apoD on endogenous apoD expression, apoptosis and proliferation. Induction of apoD mRNA (A) and protein (B) expressions was tested 3 days after the addition (+) or not (–) of 200 ng/ml of human apoD to the culture media. Northern analysis was performed with total RNAs probed with mouse apoD and GAPDH cDNAs. Western immunoblot analysis for human and mouse apoD protein was performed on total cell lysates and is representative of two separate experiments. Apoptosis (C) and proliferation (D) levels were determined by TUNEL assay and by BrdU incorporation, respectively in the presence (+) or absence (–) of 200 ng/ml of human apoD in growing cells compared with serum starved (0.2%) and LPS treated (5 µg/ml) cultures. Each bar represents the mean ratio of fluorescent apoptotic cells \pm SD in each condition of three separate experiments performed in triplicate. Asterisk indicates statistical difference compared to conditions without addition of human apoD (unpaired Student's *t*-test, **p* < 0.001).

apoD in comparison with the addition of apoDh–GFP is explained by the fact that an earlier time point is shown (3 days and 6 days post-treatment respectively).

The ability of apoD to enter the cell and its nucleus also suggests an implication in nuclear processes such as transcription activation, cell cycling or apoptosis. We investigated further the effect of extracellular apoD in growing and stressed mouse fibroblast cultures using antibodies that recognize specifically the human protein. The presence of human apoD has no influence on apoD mRNA or mouse protein expression (Fig. 8A and B), although, it can modulate cell proliferation and apoptosis. When human apoD is added to growing cells, we

observed no effect on apoptosis but a 2-fold increase in cell proliferation. Similarly, the addition of human apoD to LPS-treated cells does not affect the apoptotic rate but further increases the proliferation level already enhanced by LPS addition. However, when applied to serum-starved cells, human apoD had no effect on cell proliferation but increased apoptosis (Fig. 8C and D).

3.7. Nuclear apoD is derived from secreted protein

To further confirm that the presence of apoD in the nucleus under stress conditions is due to a reentry of the secreted protein, apoDh–GFP transfected cells were subjected to stress and to brefeldin A (BFA) treatment. Brefeldin A disrupts the structure and function of the Golgi apparatus. Immunoblot analysis of the conditioned media from apoDh–GFP transfected cells confirmed the presence of the secreted fusion protein (55 kDa) in both normal and LPS-treated cells but not in serum starved cultures (Figs. 5C and 9A). When BFA was added, apoDh–GFP disappeared from the conditioned media in both normal and LPS conditions (Fig. 9A) confirming that the apoDh–GFP detected originated from secretion via the Golgi apparatus. As expected, this inhibition of apoDh–GFP secretion abolished the nuclear localization of apoDh–GFP but caused an accumulation in the cytoplasm both in serum starvation and in LPS conditions (Fig. 9B). All together, these results strongly suggest that the nuclear apoD is derived from the secreted protein. This conclusion can be extended to the endogenous

protein since all localization studies (Figs. 5 and 6) show that apoDh–GFP behaves identically to endogenous apoD.

4. Discussion

In this study, we demonstrate that some but not all stress conditions activate apoD expression. Temperature, osmotic pressure, metal exposition and pro-apoptotic agents such as camptothecin did not affect apoD transcription. However, classical inducers of oxidative stress, H₂O₂, Rose Bengal and UV light did induce apoD mRNA accumulation in a dose-dependent manner. This response was very closely associated with a decrease in cell proliferation and viability as demonstrated with increasing concentrations of H₂O₂. Indeed, induced apoD expression was observed specifically at those H₂O₂ concentrations that caused growth arrest. This strongly suggests that the capacity of some specific stresses to trigger apoD expression is more related to their capacity to activate growth arrest pathways. The relation between apoD and growth arrest is well established. When analyzed in cell cultures, apoD induction seems always inversely correlated to cell proliferation [18–26].

This study is also the first to our knowledge that establishes a relation between apoD expression and inflammation. Indeed, the only stress that increased apoD without inhibiting cell proliferation was LPS. This molecule, derived from the outer membrane of Gram-negative bacteria, is well documented as being both a mitogen and a pro-inflammatory agonist in several cell types participating to inflammatory responses, including fibroblasts [58–60]. It is also capable to induce oxidative stress through the activation of NADPH oxidase and antioxidant enzymes. In spite of this, we believe that apoD expression is related to inflammatory pathways because of the capacity of diclofenac to reverse the LPS-associated induction. Our results also show that the LPS function on the apoD expression is mediated at the promoter level, through the APRE-3, NF- κ B and AP-1 binding sites. AP-1 and NF- κ B are well documented as regulators of numerous genes involved in immune and inflammatory responses [61]. In different cell and promoter contexts, AP-1 binds DNA as Jun/Jun homodimers or Jun/Fos heterodimers and alters gene expression in response to growth factors, cytokines, oxidative stress, and phorbol esters [62]. Less information are available concerning acute-phase responsive elements (APRE) having a consensus sequence similar to the one present on the apoD promoter. Some studies reported that APREs have an important role during inflammation and infection and to bind IL-1 α [63], IFN- γ [64], IL-6 [65] and NF- κ B1 isoforms [66]. The APRE could also bind C/EBP β which is mobilized upon LPS administration in mice. C/EBP β is an important regulator of genes involved in immune and inflammatory responses and has been shown to bind the IL-6 gene promoter as well as several other acute-phase and cytokine genes [67]. This pathway is not yet fully understood but allows the production of prostaglandin E₂ [68], a member of the pro-inflammatory eicosanoids family. Interestingly, arachidonic acid, the preferential ligand for apoD, is the dominant substrate for eicosanoid synthesis [69]. In addition to C/EBP β , the APRE

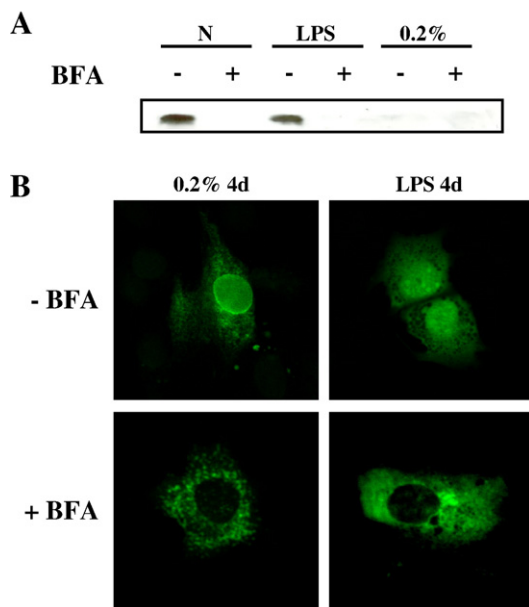


Fig. 9. Intracellular localization of apoDh–GFP in brefeldin A exposed cells. ApoDh–GFP transfected cells were subjected to growing (N), serum starved (0.2%) and LPS (5 μ g/ml) conditions during 4 days. (A) Western immunoblot analysis for human apoD was performed on the culture media of cells treated with or without BFA (5 μ g/ml) for 1 h and processed 24 h later for immunoblotting. (B) Confocal fluorescence imaging of cells transfected with apoDh–GFP, exposed to stress conditions and treated or not with BFA as in panel (A).

could also bind STAT3 [70], which is known to play important roles in cell differentiation, proliferation, survival, and angiogenesis promotion.

The accumulation of apoD under growth arrest and in presence of LPS is also observed at the protein level. However, the protein accumulation is more modest than that of the mRNA. Our results clearly show for the first time that apoD subcellular localization undergoes modifications upon stress exposure: it is mainly secreted and found perinuclear in the Golgi apparatus under basal conditions but accumulates in the cytoplasm and the nucleus in stress conditions. Perinuclear localization of apoD has been reported in oligodendrocyte precursor-like cells and astrocytes of mouse brain of Niemann–Pick type C disease model [71] and in vascular smooth muscle cells (VSMC) after wounding [72]. The accumulation of apoD in the cytoplasm and its translocation to the nucleus when growth arrest or pro-inflammatory stimuli are applied was supported by several independent experimental approaches. This included colocalization of apoDh–GFP fusion constructs with DNA and Golgi markers, immunocytochemistry of the endogenous protein, and cell fractionation. Thus, immunoblotting of apoDh–GFP transfected cells confirms that, upon serum starvation and LPS treatment, there is an accumulation of apoD both in the cell and in the nucleus. No detectable secretion in the extracellular space was obtained in growth-arrested cells although easily detectable in the extracellular space upon LPS treatment. The experiment presented the advantage that we could follow the endogenous mouse apoD and the exogenous human apoD linked to GFP. Interestingly, both the mouse and human apoD behave similarly, suggesting a common response pattern upon stress exposure including similar pathways for nuclear transport.

Nuclear apoD may have several possible sources. It is unlikely that apoD enters the nucleus by passive diffusion since the fusion protein used in our study was generated by fusing EGFP to the C-terminus of the entire coding region of human apoD. The molecular mass of the resulting fusion protein is ≈ 55 kDa and therefore, it cannot enter the nucleus by diffusion. Furthermore, apoD does not contain any common putative nuclear localization signal (NLS) that could direct its translocation to the nucleus. Still, the binding of apoD to a transmembrane receptor or a protein containing a NLS cannot be excluded. Retrograde trafficking from ER to the nucleus is a more plausible mechanism of translocating the intracellular apoD from cytoplasm to the nuclear compartment. It has been suggested that misfolded proteins that would classically undergo degradation in the ER can be back-translocated from the ER to the cytoplasm. However, if misfolding occurs, it is not the consequence of the overexpression of the apoDh–GFP since a similar localization of endogenous apoD was found in cells transfected or not with the apoDh–GFP construct. Misfolding could then be associated with the stress itself. In spite of this, we provided evidences that nuclear apoD originates from the extracellular compartment. Indeed, as a glycoprotein harboring a peptide signal, apoD should be entirely secreted. Its presence within cells exposed to specific stress suggests a protein re-uptake, most likely by an endocytosis-dependent pathway, and transport to the nucleus. This is in agreement with several

observations: (1) the apoD present in the nucleus has a molecular mass similar to that of the secreted protein; this would not be the case if apoD had bypassed the Golgi and ER glycosylation (2) apoDh–GFP is secreted as well as localized in the nucleus; (3) when untransfected stressed cells (LPS exposed or serum starved) are exposed to conditioned media containing apoDh–GFP, the fusion protein is translocated to the nucleus; (4) inhibition of protein secretion by BFA abolishes the nuclear presence of apoDh–GFP, whereas it increases its cytoplasmic content. Thus, apoD nuclear localization is dependent on the functional Golgi apparatus; (5) human apoD, purified from cyst fluid, is internalized in normal and stressed cells but translocation to the nucleus is observed only after stress; (6) finally, there is accumulating evidence that secreted proteins are present in the nucleus under specific circumstances. The fact that apoD is found in the nucleus of serum starved cells although we could not detect its presence in the media may appear as a contradiction but we cannot rule out that very low levels of apoD are secreted and immediately reabsorbed under these conditions. Indeed, the media was only concentrated twice before analysis.

Even though, apoD is translocated differentially in normal and stress conditions, its possible function in the cells remains to be determined. The data obtained by confocal imaging and immunoblotting of apoDh–GFP transfected cells and cells exposed to exogenous human apoD suggest that, in normal growing cells, apoD is modified in the Golgi apparatus and secreted before reinternalization in the cytoplasm, possibly transporting one of its ligand. The exposition of cells to exogenous apoD followed by its internalization results in an increased proliferation and points to an interaction of apoD or its ligand or both with early targets of proliferative signals. The function of apoD in the nucleus during stress is less clear. Its accumulation in the nucleus of both serum starved and LPS treated cells raised the possibility that it may regulate gene expression directly or indirectly. In particular, it suggests a role for apoD in the balance between apoptosis and cell proliferation. This balance might be dependent of the interaction of apoD or its ligand or both with cell-cycle regulators that affect both the proliferative and non-proliferative processes. Consequently, the addition of exogenous apoD has a strong incidence on cellular proliferation in LPS treated cells but apoptosis is observed when added to growth-arrested cells. Both mitogenic and growth arrest conflicting signals are known to initiate apoptosis as this could be the case in growth arrested cells treated with purified apoD. Yet, exogenous apoD does not influence endogenous apoD protein or mRNA expression, excluding the possibility of self-regulation.

Inflammation, oxidative stress and apoptosis are part of many if not all the pathological situations where apoD is found increased. Chronic inflammation is associated with certain metabolic diseases such as non-insulin-dependent diabetes mellitus type II and metabolic syndrome X (for review, see [6,43]). There is also an emerging consensus of the inflammatory and/or oxidative stress hypothesis of Alzheimer's disease, multiple sclerosis, schizophrenia, bipolar disorder, traumatic brain injury and other neuropathologies [30,44,73]. Moreover,

apoptosis was reported in situations showing apoD over-expression [37,74,75]. Thus, it might be difficult to distinguish the incidence of each of these pathways on apoD induction since most of them are intimately linked. However, the present study suggests that those conditions responsible for apoD expression are more related to an antiproliferative and inflammatory signals than to oxidation and apoptosis. It remains to be determined whether apoD accumulation in inflammation is protective or detrimental. ApoD could be part of the protective component of a mild inflammation, particularly at the neuronal level. It could also trap inflammatory mediators. In particular, apoD could trap arachidonic acid, which is released from the cellular membrane after inflammatory stimuli, and prevent its subsequent conversion in pro-inflammatory eicosanoids.

In conclusion, apoD induction is specific to ongoing cellular stress. Only pathological and/or stressful situations tested here having an inflammatory or growth arrest component have the capacity to increase apoD expression. We have provided some lines of evidence indicating that these stresses induce a displacement of apoD localization and that the nuclear apoD is derived from the secreted protein. ApoD binds several quite different molecules. Within the cell, apoD could have many functions such as the transport of specific molecules to the various compartments and evacuation of toxic metabolites. It could also play a role in the modulation of signal transduction pathways and in the regulation of nuclear processes such as transcription activation, cell cycling and apoptosis. More studies are needed to identify potential target genes of apoD and to elucidate the molecular events that trigger its cell entry and nuclear translocation.

Acknowledgments

We thank Ross Milne, Diego Sanchez and Maria Dolores Ganfornina for helpful discussions. The anti-mouse antibody was raised by Olivier Guinard in our lab. We gratefully acknowledge support from the Canadian Institutes for Health Research (Grant MOP-15677). SDC was supported by FRSQ and NSERC studentships.

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